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SHORT COMMUNICATION

Pepsin and bile acids in induced sputum of chronic cough patients

Marcin Grabowski [a,b,*], Ahmad Kasran [b], Sven Seys [b,c], Ans Pauwels [d], Wojciech Medrala [a], Lieven Dupont [c], Bernard Panaszek [a], Dominique Bullens [b,e]

[a] Department of Internal Medicine, Geriatrics and Allergology, Wroclaw Medical University,ul. Pasteura 4, 50-367 Wroclaw, Poland
[b] Laboratory of Experimental Immunology, KULeuven, Herestraat 49, 3000 Leuven, Belgium
[c] Laboratory of Pneumology, KULeuven, Herestraat 49, 3000 Leuven, Belgium
[d] Laboratory of Gastroenterological Pathophysiology, University Hospital Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium
[e] Department of Woman and Child, KULeuven, Herestraat 49, 3000 Leuven, Belgium

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Summary
One of the theories which explain, why gastroesophageal reflux disease (GORD) may provoke cough, is the occurrence of aspiration of gastric content into the airways. The aim of the study was to assess the presence of aspiration markers: pepsin and bile acids (BA) in induced sputum in gastroesophageal reflux-related (GOR-related) chronic cough (CC) patients. Forty-one CC patients and 20 healthy controls were enrolled in the study. GORD as cause of CC was diagnosed by presence of GORD-related symptoms, gastroscopy and/or improvement of cough upon treatment with proton pump inhibitors (PPI). Patients were divided into two groups based on the response to PPI treatment. In all patients and healthy controls induced sputum was obtained and differential cell counts were calculated. Levels of pepsin and BA were measured in sputum supernatants. Pepsin was detectable in 48.8% samples in CC patients and in 60% healthy controls ($p = NS$). In pepsin positive samples no significant difference in pepsin concentration could be found between CC patients and control subjects. Pepsin levels in pepsin positive samples were significantly decreased in patients treated with PPI compared to non-treated patients. BA were detectable in 56% samples of CC patients and in 70% healthy controls ($p = NS$). BA concentration in BA positive samples in CC group was not different from healthy controls. There was also no difference when comparing patients who took PPI and

* Corresponding author. Tel.: +48 602225612.
E-mail address: marcingrabow@o2.pl (M. Grabowski).

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those who did not. Patients characterized as PPI-responders and PPI-non-responders had similar pepsin and BA concentrations. Airway cellularity was not significantly different between groups of patients with or without pepsin or BA in induced sputum. Our results demonstrated the lack of differences in gastric content aspiration between patients with probable GOR-related CC and healthy control subjects. This might imply that the reflex cough theory may be more relevant than the reflux-associated aspiration theory in the pathophysiology of GORinduced chronic cough.

Introduction

It is well documented that gastroesophageal reflux disease (GORD) and cough often coexist. Cough in subjects with GORD can be evoked by two possible mechanisms: a broncho-oesophageal neurogenic reflex, or aspiration of gastric content into the airways (true "reflux" theory). The microaspiration theory is supported by an observed fall in tracheal pH following gastroesophageal reflux event and by the presence of aspiration markers in bronchoalveolar lavage fluid (BALF).[1] Pepsin is a good marker of acidic gastroesophageal reflux, and its presence in sputum or saliva is considered to be pathologic. Its enzymatic activity in spontaneous sputum and saliva might be a better test for reflux-related aspiration than 24-h oesophageal pH monitoring and the pepsin assay could also be used to evaluate reflux events without interrupting therapy with H2-blockers or proton pump inhibitors (PPI).[2] Bile acids aspiration has been associated with pulmonary injury[3] with dose-dependent cytotoxicity ranging from alteration of cellular cationic permeability to disruption of the cellular membrane.

Sputum induction, sampling the central airways as a non-invasive method to study airway inflammation and to monitor airway diseases, is well established and routinely used.

The aim of this study was to assess the 'true reflux theory' in chronic cough patients by studying the occurrence of gastric content aspiration in the airways by measuring pepsin and bile acids levels in induced sputum and assessing their relationship with induced sputum cellularity.

Patients and methods

Study subjects were recruited between November 2009 and February 2010 at the Department of Internal Medicine, Geriatrics and Allergology of Wroclaw Medical University. Patients referred to the outpatient clinic with a history of chronic cough (lasting at least 8 weeks) were consecutively included after signing informed consent. A diagnostic protocol according to ERS guidelines was performed in all patients.[4] Patients with postnasal drip syndrome were included if they had negative plain sinus radiography and did not respond to previous treatment with topical corticosteroids or antihistaminics. Other exclusion criteria were: (1) any abnormalities in chest radiograph, (2) asthma (reversibility to bronchodilators or a positive histamine provocation test), (3) smoking history of more than 5 pack-years, (4) a respiratory infection within 4 weeks of inclusion, or (5) cough induced by angiotensin converting enzyme inhibitors (iACE). The control group consisted of healthy non-smokers with negative allergic history, no clinical symptoms of upper or lower airway disease, and no typical GORD symptoms. Informed consent was obtained from all study subjects. The study was approved by the local Ethical Committee of Wroclaw Medical University, Poland.

Forty one patients with chronic cough (11 males, mean age 44 years (21—75y)) and 20 control subjects (7 males, mean age 44 years (24—66 years)); $p = 0.72$ and 0.74 for sex and age respectively, were included.

Mean cough duration in patients was 3.9 years (0.5—20y). Mean FEV1% predicted, FVC% predicted and FEV1/FVC in patients were respectively 108.6% (84.7—136.8%); 105.3% (79.6—123.0%) and 103.2 (87.0—117.4).

We first evaluated the presence of GORD in our study subjects, based on the patient's medical records and by means of a detailed patient interview.

The presence of typical (heartburn, retrosternal pain, belching) or atypical GORD symptoms, the results of gastroscopy and details on the acid suppression treatment were recorded. None of the subjects in the control group revealed any GORD symptoms. At the time of sputum induction 12 patients were already treated by PPI (omeprazole 40 mg or pantoprazole 80 mg daily) (see Table 1).

After sputum induction all patients were uniformly treated with omeprazole 40 mg per day ($n = 39$). The response to PPI treatment was assessed 4 weeks later. A positive response to PPI treatment was defined as a partial or complete resolution of cough ($n = 15$). When cough did not improve sufficiently after at least 4 weeks treatment with PPI, patients were scored to have a negative response to PPI ($n = 12$).

Methodology of sputum induction, processing and cytospin preparation has been published previously.[5]

Pepsin levels in sputum supernatants were measured by ELISA kit (USCN Life Science Inc. Wuhan, China) by adding the same concentration of DTT (0.1%) to the standard curve samples as used in the sputum supernatants (detection limit was 1.56 ng/ml). Total bile acids (TBA) concentration in

Table 1 Distribution of patients treated with PPI at the time of sputum induction and their response to PPI.

Number of patients		PPI response		
		Positive	Negative	
PPI at the time of sputum induction	Yes	9	3	12
	No	15	12	27
		24	15	

Table 2 Concentrations of pepsin and bile acids in different study subgroups.

	Pepsin [ng/ml]	Bile acids (BA) [μmol/l]
Chronic cough ($n = 41$)	6.4 ± 6.4	0.5 ± 0.2
Healthy controls ($n = 20$)	7.3 ± 7.2	0.5 ± 0.2
PPI treated at the time of sputum induction ($n = 12$)	8.6 ± 6.8	0.4 ± 0.2
PPI non-treated at the time of sputum induction ($n = 27$)	2.5 ± 2.6	0.5 ± 0.2
Reflux oesophagitis (RO+) ($n = 27$)	6.7 ± 7.6	0.5 ± 0.3
Without reflux oesophagitis (RO-) ($n = 10$)	6.1 ± 4.5	0.4 ± 0.2
PPI responders ($n = 24$)	5.8 ± 5.6	0.5 ± 0.3
PPI non-responders ($n = 15$)	7.6 ± 8.0	0.4 ± 0.2

sputum supernatants was analysed by enzymatic assay (Bioquant, San Diego, USA) (detection limit was 0.2 μmol/L).

Data are presented as median and interquartile range, or as mean and standard deviation, where appropriate. Differences between the patients and controls were estimated by Mann-Whitney-U or by the Kruskal–Wallis test (for more than 2 groups). Correlations were verified by Spearman correlation coefficients (r). A probability of $p < 0.05$ was regarded as significant.

Results

In 37 out of 41 patients gastroscopy was performed. In 27/37 patients reflux oesophagitis (RO) grade A ($n = 26$) or B ($n = 1$) was diagnosed based on the Los Angeles classification.[6] All 41 patients had at least one of the GORD-related typical symptoms and aggravation of cough accompanying activities resulting in lower oesophagus relaxation, like change in posture, during/after meals, or physical activity. The exclusion of other causes of cough and the presence of oesophagitis, or typical symptoms suggesting GORD in all of the CC patients included in our study, suggests that all patients had a diagnosis of gastroesophageal reflux-related cough (GOR-related cough).

Positive response to PPI was diagnosed in 24 patients, 15 patients did not improve with PPI ("PPI-non-responders" or "chronic idiopathic coughers (CIC)", see Table 1). In 2 patients the response to PPI was not assessed. PPI responders and non-responders (CIC) did not differ in age (44.8 vs 42.5 years, $p = 0.7$); gender (males: 8 vs 3, $p = 0.6$), duration of cough (3.6 ± 5.0y vs 4.8 ± 4.8y, $p = 0.26$) or lung function parameters: FEV1% predicted (107.6% ± 11.1 vs 109.0% ± 15, $p = 0.9$).

Pepsin was detected in 20 out of 41 (49%) sputum samples from chronic cough patients and in 12/20 (60%) samples from healthy controls (p = NS). In pepsin positive samples ($n = 20$), no significant difference in pepsin concentration was observed between chronic cough patients and control subjects ($n = 20$) (p = NS). Induced sputum pepsin levels in pepsin positive samples were significantly decreased in chronic cough patients treated with PPI at the time of sputum induction, when compared to those chronic cough patients without PPI treatment or healthy controls ($p = 0.018$, Table 2, Fig. 1a). Pepsin concentrations in induced sputum were not significantly different between chronic cough patients with reflux

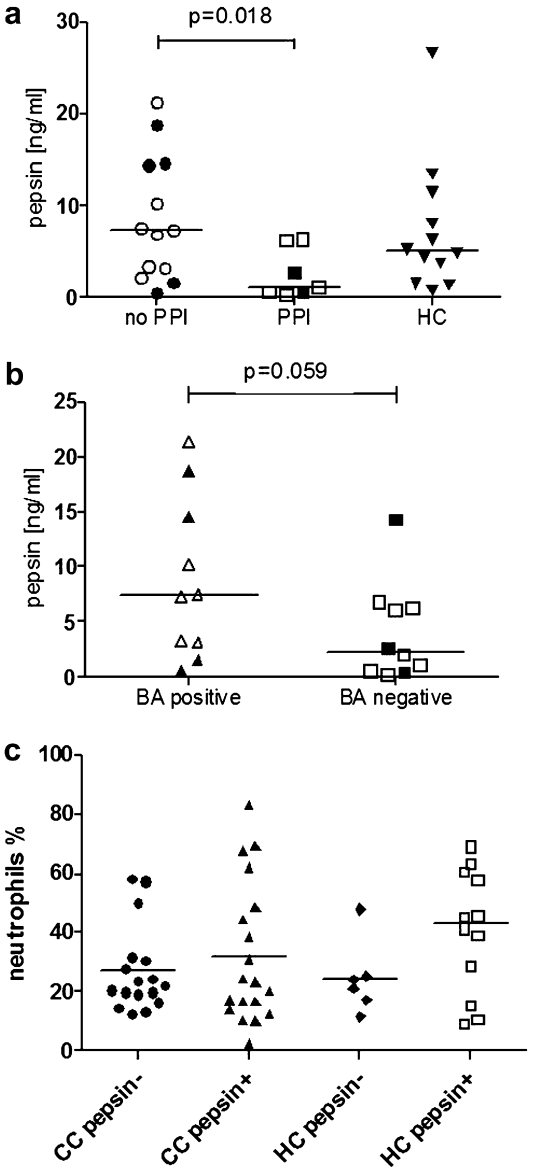

Figure 1 Pepsin concentration in pepsin-positive samples in chronic cough patients, treated with PPI, not treated with PPI, and healthy control subjects (a) and in samples negative and positive for BA (b); as well as the percentage of neutrophils in patients and healthy controls with or without detectable pepsin in induced sputum (c). Sputum was induced in 41 patients with CC and 20 HC. Pepsin (a, b, c) and BA (b) concentrations were measured by ELISA and enzymatic assay respectively. White blood cells were counted on cytospin and the proportion of neutrophils is represented in percentage (c). In a and b, only pepsin-positive samples are represented. PPI-responders are represented by closed symbols, non-responders - by open symbols (a, b). The median is indicated by a horizontal line. Comparisons were performed using two-tailed Mann–Whitney U-test (b) or Kruskal–Wallis test (a, c).

Table 3 Differential cell count in chronic cough patients (CC).

		[a]CC, pepsin+	CC, pepsin−	HC	*p*-value
Absolute	Total cell count	3.3 ± 1.8	2.6 ± 1.5	3.8 ± 2.5	0.26
Relative[b]	Epithelial cells %	37.6 ± 17.5	40.7 ± 14.9	47.9 ± 17.0	0.52
Relative[c]	Neutrophils %	32.3 ± 23.8	27.2 ± 14.5	36.0 ± 19.8	0.43
	Macrophages%	65.7 ± 23.8	71.6 ± 15.5	61.9 ± 20.6	0.41
	Eosinophils%	0.6 ± 0.9	0.2 ± 0.4	0.7 ± 1.0	0.17
	Lymphocytes%	1.5 ± 1.5	0.9 ± 1.2	1.5 ± 1.1	0.12
Absolute	Neutrophils	1.3 ± 1.6	0.7 ± 0.5	1.6 ± 1.4	0.29
	Macrophages	1.9 ± 0.9	1.9 ± 1.2	2.1 ± 1.5	0.77
	Eosinophils	0.02 ± 0.03	0.006 ± 0.01	0.02 ± 0.04	0.17
	Lymphocytes	0.05 ± 0.06	0.02 ± 0.02	0.06 ± 0.07	0.13

[a] Chronic cough patients were subdivided into pepsin positive (pepsin+) and pepsin negative (pepsin−) patients, and compared to healthy controls (HC). Comparison was done using Kruskal–Wallis test.
[b] Cytospins were prepared, 250 cells were counted and the percentage of epithelial cells was studied.
[c] Cytospins were prepared, 250 white blood cells were counted and the relative proportion of neutrophils, macrophages, eosinophils and lymphocytes was studied.

oesophagitis (RO+) and those without (RO-) or between PPI-responders and PPI-non-responders − Table 2.

Low levels of BA were detected in 23 out of 41 (56%) samples of chronic cough patients and in 14/20 (70%) samples of healthy controls (p = NS). In BA-positive samples, no significant difference in total bile acid concentration was observed between chronic cough patients and control subjects (p = NS**).** In contrast to what we observed for pepsin, BA levels did not vary between PPI treated and untreated patients. As we observed for pepsin measurements, sputum BA levels were comparable between RO+ and RO- patients and between PPI responders and non-responders, (see Table 2).

Pepsin concentrations tended to be higher in chronic cough patients with BA in induced sputum: 8.8 ± 7.3 vs. 4.0 ± 4.4 ng/ml, respectively ($p = 0.059$; Fig. 1b). However, no significant correlation was found between BA and pepsin levels in induced sputum ($r = -0.25$, $p = 0.33$).

Induced sputum differential cell count was not significantly different between chronic cough patients and the healthy control subjects, see Table 3. Similar results were observed when comparing total and differential cell count in patients with or without measurable pepsin in induced sputum, although there was a trend towards increasing percentages of neutrophils in induced sputum when sputum pepsin was detectable (Fig. 1c).

Discussion

Patients with chronic cough, suspected to have gastro-esophageal reflux with or without improvement of cough upon PPI treatment, do not appear to have more features of microaspiration than control subjects. In agreement with these findings, no or very low levels of pepsin or bile acids were detected in BAL or saliva in chronic cough populations studied by Stavold et. al and Blondeau et al.[3,7] The low levels of pepsin in subjects/controls could partially be affected by pretreatment with dithiothreitol (DTT), when dissolving induced sputum. DTT has some protease activity but the impact on pepsin has not been evaluated yet.[8] Furthermore, Ervine and coworkers recently showed significant increase of pepsin in saliva from healthy children after sputum induction, which could be caused by the procedure itself.[9]

In accordance to previous observations by Patterson et al.,[10] we found similar differential cell counts in induced sputum samples from chronic cough patients with or without measurable sputum pepsin or BA. In contrast, studies by Carpagnano et al.[11] demonstrated increased neutrophilic airway inflammation in patients with GOR-related chronic cough. Relative neutrophil counts in our study also tended to be higher in pepsin-positive samples obtained from both study and healthy subjects when compared to pepsin-negative samples (Fig. 1c). Pepsin or BA concentrations in induced sputum did not correlate with age, duration of cough, spirometric parameters, or airway cellularity (data not shown).

In conclusion our findings of similar pepsin and BA levels in induced sputum of chronic cough patients and healthy control subjects argue against the occurrence of significantly more gastric microaspiration in GOR-related chronic cough patients when compared to healthy controls. This was corroborated by the absence of significant differences in pepsin and BA levels when these patients were compared on the basis of presence/absence of reflux oesophagitis or response/nonresponse to PPI. These findings do not exclude GORD as a potential cause of chronic cough but suggest that the 'cough reflex theory' might be more relevant than the 'reflux-aspiration theory' to explain the pathophysiology of GOR-induced chronic cough

Conflict of interest

None declared.

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